GENE EDITING FOR CANADIAN FIELDS CROPS – TARGETS AND BENEFITS

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Executive Summary

With the fast-growing global population, which is projected to reach 9.7 billion people in 2050, there will be an unprecedented demand for food crops, animal feed and biofuel. However, global crop yields are plateauing as a result of unconducive cyclical weather patterns, and increased spread of diseases and pests. The vanishing of biodiversity of ecosystems and diversity of agricultural crops along with loss of arable land has increased the demand for more sustainable and precise agricultural practices. A major challenge for plant breeders is developing environmentally resilient cultivars that perform in a stable manner in response to changing agronomic practices and variable environments conditions resulting from climate change. Plant breeders rely on genetic variability, breeding tools, and methods to rearrange desirable genes into commercial cultivars – largely focusing on traits such as higher yield, disease resistance, abiotic stress (heat, drought, cold) tolerance, and improvement of nutritional quality of foods for a growing world population. Pathogens damage crops and rob yield potential, and an important component of plant breeding is to stay ahead of this curve, by identifying and incorporating durable disease resistance to protect the crop without the need for chemical control of pathogens.

Crop breeding has benefited from modern technologies to support cultivar development. This is particularly true in case of some of the world’s most important crops – for example, maize, wheat and rice. Molecular markers have increased breeding efficiency by selection of traits based on genomic information rather than by phenotyping. However, despite the benefit of molecular tools and genomic information, combining multiple desirable agronomic outcomes is still hindered by genetic correlations between traits. The rapid development of CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR associated proteins)-based gene editing has created an alternative avenue for crop improvement and has the potential to increase speed and precision in plant breeding programs. Due to its high precision, ease of design, multiplexing ability, and low cost, this innovative technology has the potential to revolutionize agriculture. Here, we summarize examples of the integration of CRISPR/Cas-based gene editing into to create varieties for various applications and growth environments. We
highlight the use of CRISPR/Cas-based gene editing to accelerate *de novo* domestication, generate novel/superior alleles in elite lines, and “promoter bashing” for increased resilience of crops to abiotic stresses. While our examples have been abbreviated, a more detailed review has been published recently (Lyzenga et al. 2021). Furthermore, we summarize how CRISPR/Cas9 gene editing is being used to engineer durable disease resistance to various pathogens and pests, to increase quality and nutritional traits of new crop varieties and to eliminate ‘anti-nutritional’ compounds in crop plants. While survey results reveal a consensus on the potential of gene-editing on crop improvement, health and safety regulations, followed by export markets, consumers, and the media play a major role in determining its adoption on a larger scale.

1. **Fixing Desirable Single Gene Traits in Breeding Programs**

Some plant traits are under simple genetic control, usually being regulated by only one or a few genes. In some cases, these genes have been sequenced and their functional influence on trait expression has been confirmed. For example, in durum wheat, the *Cdu-B1* gene controls cadmium (Cd) accumulation in durum wheat grain, and the low Cd allele codes for a heavy metal transporter that locks the Cd in root cells, preventing the harmful heavy metal from accumulating in the grain. High accumulation results from a small DNA insertion into the gene, rendering it ineffective in sequestering Cd into roots. In this case, Cd freely moves and translocates to the grain. Gene editing can be used to precisely convert this undesirable allele of *Cdu-B1* into favorable low Cd allele in germplasm. Knowing the function of this heavy metal transporter provides an opportunity where gene editing technology could be used to edit similar genes in other crops that accumulate Cd, such as in Flax. Gene editing could be coupled with rapid-generation cycling to quickly produce productive lines with desirable alleles.

There are several other examples of monogenic improvement traits which have functionally characterized genes/alleles. For example, the wheat dwarfing alleles Rht-B1b (formerly Rht1) and Rht-D1b (Rht2) each contain a single base-pair mutation which gives rise to a premature STOP codon, resulting in a truncated protein with altered function in gibberellin signal transduction. The dwarfing alleles confer reduced plant height, resulting in increased harvest index. Similarly, the null allele of grain weight 2 (GW2), a regulator of cell division, leads to an increase in grain
width and weight in rice and wheat (Song et al., 2007; Simmonds et al., 2016; Wang et al., 2018; Zhang et al., 2018). The dwarfing and GW2 alleles could be precisely targeted by gene editing in breeding programs. By fixing a collection of monogenic traits plant breeders could phenotype and perform selections from a population of plants which already have a basic complement of non-segregating traits, such as plant height and grain weight. Plant breeders would thus have more resources to explore and select for other important and more genetically complex traits.

Furthermore, gene editing can be used to save near-miss varieties. Often during the 8-12 year breeding process, new varieties are developed which exhibit many desirable features such as high yield and superior end-use quality/nutrition. However, unanticipated changes, like changes in a pathogen population or disease susceptibility negatively associated with some agronomic trait may impact the viability of the new variety. For example, in wheat resistance to Fusarium head blight (FHB) is highly desirable; however, susceptibility to FHB is associated with the semi-dwarfing stature, perhaps due to linkage. While FHB resistance is multifaceted, and thus more challenging for gene editing experiments, semi-dwarfing allele is simple and thus could be the target of gene editing technology. Gene editing technology provides a faster and precise approach to generate desirable alleles of dwarfing genes into the otherwise agronomically superior cultivars.

2. Gene Editing to Promote Domestication

Domestication followed by intensive breeding have resulted in a genetic bottleneck and many modern crop germplasms have genomic regions of reduced genetic diversity (Shi and Lai, 2015). Trait variation from landraces and wild relatives represent a rich reservoir of genetic variation that can be introduced through introgression breeding. However, this process is tedious and time consuming. Incorporating the beneficial allelic variation into elite lines and leaving behind unadapted genetic material is a major challenge and may not be successful because of introgression across species barriers. However, CRISPR/Cas based gene editing has emerged as tool for generation of novel and superior alleles within crop germplasm or within elite lines (Rodríguez-Leal et al., 2017; Nogue et al., 2016; Shen et al., 2017). In contrast to random mutagenesis through ethyl methanesulphonate (EMS) and gamma irradiation, CRISPR/Cas based
gene editing can be targeted to genomic regions of interest such as promoters, developmental regulators, and transcription factors to promote semi-random mutagenesis. Since CRISPR/Cas based gene editing can be easily multiplexed, multiple genetic regions can be targeted at once.

Recent technological advances have raised the possibility of de novo domestication (i.e., the introduction of domestication genes into non-domesticated species) of wild plants as a viable solution to promote the use of wild relatives in plant breeding (Fernie and Yan 2019). Indeed wild relatives of modern and orphan crops were mainly regarded as a source of novel genetic variation particularly for disease resistance. However, traits such as small fruit size, low yield, excessive height and seed dispersal resulting from shattering, constrain their use in breeding programs targeting commercial varieties. Recently, the concept of de novo domestication through gene editing has been explored as an exciting possibility to “adapt” wild germplasm by editing genes influencing these undesirable characteristics (Zsögön et al., 2017; 2018). This is plausible using a CRISPR/Cas9 gene editing platform because most domestication genes are well characterized and have simple genetic architecture. There are several examples of how domestication genes were targeted in biotechnology approaches that predate the development and widespread adoption of genome-editing techniques (Østerberg et al. 2017). The use of CRISPR technology has recently been demonstrated in tomato (Li et al. 2018b; Zsögön et al. 2018), where simultaneous editing of six loci important in domestication resulted in increased fruit number (MULT), size (FW2.2, FAS), shape (O gene; OVATE), nutritional content (LYCOPENE BETA CYCLASE) and plant architecture (SP gene; SELF-PRUNING) (Zsögön et al. 2018). Similarity, domestication genes impacting day-length insensitivity (SP5G), fruit size (SICLV3, SIWUS), vitamin C levels (SIGGP1) and plant architecture (SP) were stacked in accessions of Solanum pimpinellifolium with disease and salt tolerance (Li et al. 2018b). These studies demonstrate that CRISPR/Cas based gene editing can accelerate domestication and increase the value and use of orphan crops or wild relatives in plant breeding.

3. CRISPR/Cas9 for Generating Novel and Superior Alleles

Most traits targeted by breeders for improvement are controlled by multiple genes, where expression is quantitative, and influenced by the environment. For example, resistance
to cold tolerance in cereal crops is genetically complex, and multiple genes have been implicated in improved cold tolerance. Many studies support that the relative level of gene expression of many interacting genes regulates the cold tolerance pathway – meaning there are multiple genes, each regulated differently, depending on environment. This creates a challenge for gene-editing as it is not always clear “which gene should be edited”, and “what should the precise edit be”. One approach to deal with these complex traits via gene editing as a tool to develop superior alleles within crop germplasm or within elite lines is to use “promoter bashing” (Nogué et al. 2016; Rodríguez-Leal et al. 2017). Cis-regulatory elements (CRE) are in part responsible for regulating gene expression and have emerged as ideal regions to target via gene editing to generate a range of phenotypes (Swinnen et al., 2016). CRISPR/Cas9-induced changes in CRE generally results in spatial and temporal changes in gene expression resulting in changes in quantitative trait variation (Rodríguez-Leal et al. 2017; Swinnen et al. 2016). Using this approach, a range of fruit size in tomato was achieved through semi-random CRISPR-induced mutations in regulatory region of the CLAVATA (SlCLV3) gene (Rodríguez-Leal et al., 2017). This approach can be applied to many species to generate allelic series for a variety of traits relevant to plant breeding. A similar approach has been applied to the protein coding regions to achieve directed evolution for engineering improved or new functions in plants (Butt et al., 2019).

Abiotic stress tolerance is governed by several genes and largely influenced by environmental factors which make it challenging to study (Bhat et al. 2016; Deshmukh et al. 2014) and very difficult to develop new cultivars using conventional methods. CRISPR/Cas9 has potential to alter the stress response of crop plants. Shi et al. (2017) have developed a corn variety through CRISPR/Cas genome editing approach, which has improved yield under drought stress. In another study, a tissue-specific AtEF1 promoter was used to drive CRISPR/Cas9 induced mutations in abiotic stress-responsive genes, leading to enhanced stomatal responses (Osakabe and Osakabe 2017). Rice genes OsRR22 and OsNAC041 have also been targeted to increase salinity tolerance (Bo et al. 2019; Zhang et al. 2019). Regulatory sequences in promoters, such as W-box, GCC box (AGCCGCC), MYBR or DBS (TGCTANNATTG), which function as negative regulators of abiotic stress response by providing binding sites for transcription factors, could also be targeted by gene editing (Zafar et al., 2019).
Another approach to enhance abiotic stress tolerance is to modify the micro-structure of DNA through induced changes in DNA methylation. Reducing methylation of a gene greatly impacts its activity and function (Maurano et al. 2015). Therefore, it is possible to elicit gene expression by demethylating components of the promoter region. Many epigenetic factors, such as H3K4me3 and H3K9ac, have been associated with abiotic stress tolerance, including heat and drought stress tolerance in maize, and for soil salinity tolerance in wheat (Varotto et al. 2020). These epigenetic factors represent potential targets for gene editing technologies for creating crop varieties adapted to specific environments. Alternatively, synthetic regulatory elements could be inserted into promoters to enhance expression of abiotic stress resistance genes, but this approach would require precise knowledge of specific genes that regulate the stress response.

4. Modulating Gene Recombination in Plants Using Gene Editing

Genetic recombination plays a foundational role in plant breeding as it allows for allele reshuffling and creates novel allelic combinations. It plays a critical role in introgression of a beneficial locus from a donor line into an elite line through backcrossing (Moose and Mumm, 2008). Recombination frequencies can be increased by using of chemical agents or physical stress, such as temperature shock or UV exposure (Wijnker and de Jong 2008), but results are often unpredictable. Ideally, backcrossing would result in a progeny containing just a small introgression from the donor line, but this is rarely the case, resulting in larger introgressions that may have a negative impact on one or several unrelated traits (often referred to as linkage drag). This is because recombination is not evenly distributed along chromosome and generally occurs in regions termed hotspots and is suppressed in other regions. For example, in wheat crossover events mainly occur at the distal region of both arms of the chromosomes while recombination is largely absent in the centromere proximal region (Choulet et al., 2014; Gardiner et al., 2019). Given that genetic recombination defines the amount of genetic diversity accessible to breeders, manipulation of recombination frequency is the focus of immense study. Because of its ability to target specific genomic regions and ability to generate double stranded DNA breaks (a prerequisite for genetic recombination), CRISPR/Cas gene editing is beginning to be used to
promote recombination at specific genomic regions (Filler Hayut et al., 2017; Sarno et al., 2017). In yeast, the left arm of chromosome 7 was targeted with 95 gRNAs to induce mitotic recombination (Sadhu et al., 2016). In tomato genomic sections of linked loci represent approximately 25% of the assembled genome (Lin et al., 2014). This is a prime example of where CRISPR/Cas based gene editing could be used for generation of recombinant individuals, generating diversity and breaking up these genetic linkages that would otherwise be not possible. However, work remains to identify the specific targets for editing to achieve the desired response.

5. Durable Crop Disease Resistance

Plants are constantly infested by a variety of pathogens, including viruses, bacteria, and fungi (Taylor et al. 2004), that can cause significant losses of crop quality and yield (Savary et al. 2012). Genome engineering technologies have been widely harnessed to engineer plant resistance against pathogens (Ali et al. 2015; Baltes et al. 2015; Iqbal et al. 2016; Ji et al. 2015). CRISPR/Cas9 and TALEN (transcription activator-like effector nuclease) were successfully used to generate resistance to powdery mildew by simultaneously targeting the three homologs of the MILDEW-RESISTANCE LOCUS (MLO), TaMLO-A, TaMLO-B, and TaMLO-D, in wheat (Wang et al. 2014). The Tomelo transgene-free tomato, which is resistant to powdery mildew disease was developed by targeting the SlMlo1 gene using CRISPR/Cas9 (Nekrasov et al. 2017). Simultaneous modification of the three homologs of the wheat TaEDR1 gene enhanced resistance to powdery mildew disease (Zhang et al. 2017a). In other efforts, knockout of the ethylene-responsive factor (ERF) gene OsERF922, a negative regulator of rice blast resistance, enhanced resistance to the blast fungal pathogen (Wang et al. 2016a). Considerable knowledge has been accumulated on the genetic basis of plant disease resistance, and genes related to disease resistance have been identified in different plant species, including Arabidopsis, rice, soybean, potato and tomato (Hammond-Kosack and Jones 1996; Michelmore 1995). The wheat gene resistance allele Lr34(res) provides durable resistance against several pathogens, including two rust diseases, leaf rust and stripe rust, as well as powdery mildew and has been widely used in wheat breeding programs (Kolmer et al. 2008). The resistant allele of Lr34(res) differs from the susceptible allele
by genetic polymorphisms which change two amino acids in predicted transmembrane helices of an ABC transporter, a large family of genes in wheat (Krattinger et al. 2009; Risk et al. 2012). Fixing this allele using gene editing would greatly benefit subsequent breeding programs. Similarly, other members of the Lr family could be potential targets for gene editing technologies to produce plant immunity to various pathogens.

Pathogens exploit plants’ susceptibility (S) genes to facilitate their proliferation (Pavan et al. 2009). Disrupting these S genes may interfere with the compatibility between the host and the pathogens and consequently provide broad-spectrum and durable disease resistance (Zaidi et al. 2018). Recent studies have demonstrated the effectiveness of new transgene-free gene editing technologies for deleting S genes in various economically important crops, including wheat (Shan et al. 2013; Wang et al. 2014), rice (Jiang et al. 2013; Li et al. 2012), tomato (Paula de Toledo Thomazella et al. 2016). Disruption of the S genes (Mlo, mildew resistance locus O) has conferred powdery mildew resistance in field for more than seven decades (van Schie and Takken 2014). Mutation of Mlo with CRISPR/Cas9 has also conferred powdery mildew resistance in wheat (Nekrasov et al. 2017; Shan et al. 2013; Wang et al. 2014) and tomato (Nekrasov et al. 2017). Another powdery mildew susceptibility locus, enhanced disease resistance 1 (EDR1), has been targeted by CRISPR/Cas9 and resulted in significant reduction of powdery mildew in wheat (Zhang et al. 2017b). Taken together these studies illustrate the efficacy of gene editing platforms in the development of disease-resistant crop varieties by introducing site-specific mutations to disrupt S genes in a transgene-free manner.

Plant pathogens can evolve rapidly in agriculture, especially in the presence of genetically uniform species and monocultures grown on a large scale, resulting in the break down of resistance that has already been deployed in commercial varieties. Breeders of all crop kinds must stay “ahead of the pathogen” by identifying multiple sources of effective resistance that are then combined or “stacked” together in an elite variety. In some instances however, only a single known effective resistance gene is available. For example, antibiosis resistance to the orange wheat blossom midge of wheat is conferred by the Sm1 gene. Sm1 is the only described OWBM resistance gene (Thomas et al. 2005), and efforts to identify and incorporate additional sources of resistance are ongoing (Thambugula et al. 2021). If Sm1 resistance were to
breakdown, gene editing could be used to introduce “soft”, single-point mutations that could partially, or fully restore the resistance function, a process referred to R-gene recovery (Jang et al. 2020). Indeed, this would require substantial understanding of the Sm1, its function in the wheat plant in sensing that insect and initiating a defensive response. Fortunately, the Sm1 gene was recently cloned (Walkowiak et al. 2020), providing an opportunity to understand the functional basis of resistance in wheat.

6. Reducing Gluten Immunogenicity AND IMPROVING GRAIN QUALITY in Wheat

Whole grain foods, including wheat, that contain all parts of the grain (i.e., the bran, starchy endosperm, and the germ) are known for their health benefits, reducing the risk of several non-communicable diseases (Ross et al. 2017; Zong et al. 2016). However, the ingestion of gluten proteins (gliadins and glutenins) from wheat, barley and rye can cause coeliac disease (CD) in genetically predisposed individuals (Gujral et al. 2012). CD leads to malnutrition and various related symptoms, ranging from bowel disorders to skin-, bone-, nerve-, and muscle-problems. The only way to prevent CD is a gluten-free (GF) diet, requiring complete exclusion of wheat, barley and rye. This is very difficult to adhere to, as gluten (especially from wheat) is added to many processed food products due to its viscoelastic and binding properties (Atchison et al. 2010).

Bread wheat contains two groups of gluten proteins: glutenins and gliadins. Both gliadins and glutenins contain immunogenic epitopes within their protein sequences that cause CD (Mitea et al. 2010a; Salentijn et al. 2009). So far, no food processing or breeding strategies have been developed that produce wheat-based food products that approach safety for CD patients (Boukid et al. 2017; Jouanin et al. 2018; Rustgi et al. 2019), although there is a clear need to do so (García-Molina et al. 2017; Ribeiro et al. 2018). With the ultimate goal of removing the immunogenic gluten epitopes from the human diet, CRISPR/Cas9 technology is being used in the development of wheat lines with fewer gluten genes and/or gluten genes with inactivated CD epitopes. As proof of concept, CRISPR/Cas9 technology has been used to edit α-gliadin genes (Sánchez-León et al. 2018) as well as both α- and γ-gliadin genes (Jouanin et al. 2019a; Jouanin et al. 2019b; Jouanin et al. 2020) in bread wheat. Along with ω-gliadins, these gliadin types rank
highest in abundance and overall immunogenicity compared with the low molecular weight (LMW) and high molecular weight (HMW) glutenins (Sollid et al. 2012; Tye-Din et al. 2010). α- and ω-epitopes are highly homologous (Dahal-Koirala et al. 2019; Tye-Din et al. 2010). With CRISPR/Cas9 it is possible to edit all gliadin epitopes by causing local deletions and frameshifts. However, after the first round of edits, modifying the few remaining epitopes in some of the genes would require multiple rounds of additional edits. Specific amino acid substitutions in a gliadin epitope can abolish its immunogenicity (Mitea et al. 2010b) while having no effect on gene expression and thus on bread dough quality.

Depending on intended end-use, gene editing could be used to develop a grain quality package consisting of multiple desirable alleles. For example, grain hardness can be addressed by editing the puroindoline-A and puroindoline-B (PIN) genes (Nadolska-Orczyk et al., 2009; Matus-Cadez et al. 2008). Wheat protein content can be improved by a single base pair edit in the TaNAM-B1 gene, a NAC transcription factor found in the Gpc-B1 locus (Uauy et al., 2006). Fixing these alleles through gene editing would reduce or eliminate the need for selection for these desirable alleles.

7. Gene Editing for Improved Quality of Barley

In Canada, about eight million tonnes of barley are produced each year. It is used for human food, livestock feed, and as malt to brew beer. One of the more beneficial substances in barley is beta-glucan (Limberger-Bayer et al. 2014). Barley cultivars with high grain beta-glucan content are preferred by the food sector because of its beneficial effect on human health (Cavallero et al. 2002; Collins et al. 2010). The beta-glucan acts as fermentable dietary fibre that reduces the risk of diet-related conditions such as cardiovascular disease, type II diabetes and colorectal cancer (Ames et al. 2019; Cosola et al. 2017) and contributes to lower cholesterol (Sima et al. 2018). However, the brewing and distilling industries require barley cultivars with low grain beta-glucan content for efficient malting and brewing (Bamforth and Martin 1983; Izydorczyk and Dexter 2008). This is because beta-glucan content has a direct impact on the viscosity of the mash and if too high, leads to filtration problems during brewing or to the formation of undesirable hazes in the final product (Gupta et al. 2010).
Scientists have used CRISPR/Cas9 technology to change beta-glucan levels in barley (Garcia-Gimenez et al. 2020). The authors used reverse genetics approach to generate changes in members of the gene superfamily responsible for making beta-glucan. Not only this approach allows improving grain quality, but researchers can also introduce some specific changes that would be desirable for specific industries that use barley. In other words, plant breeders will be able to create specialized barley cultivars for beer, bread, and other uses. The lignocellulosic residues of barley have the potential to be used as a feedstock for various purposes, including biofuel production. However, its heterogeneous properties and intrinsic recalcitrance caused by cell wall lignification have lowered the biorefinery efficiency. Lee et al. (2021) were able to reduce lignin content in barley by CRISPR/Cas9-mediated mutagenesis of caffeic acid O-methyltransferase 1 (HvCOMT1), the lignin biosynthetic gene responsible for lignin formation. The mutant had 14% lower total lignin content and 34% higher fermentable glucose recovery rate, compared to the wild type. Thus, the transgene-free HvCOMT1 mutant barley could offer improved quality lignocellulosic feedstock for efficient lignocellulosic biofuel production.

8. INCREASED Nutritional Value of Canola Oil and Seeds, using CRISPR/Cas9.

Since their domestication, Brassica oilseed species have undergone progressive transformation in parallel with breeding and molecular technologies. The canola (Brassica napus) crop has rapidly expanded globally in the last 30 years with intensive innovations in canola varieties, providing for a wider range of markets. Canola is the second most important oilseed crop in the world, ranking only behind soybean in production and value (Foreign Agricultural Service/USDA, 2020). Breeding efforts of canola have been mainly focused on improving seed yield, oil quality, and meal quality along with disease resistance, abiotic stress tolerance, and herbicide resistance (Ton et al. 2020). The parental lines for rapeseed breeding programs varied depending on the geographic location, with the spring type being widely cultivated in Canada, Australia, and northern Europe, the winter type being predominant in Asia and the remaining area of Europe (Heslop-Harrison 2013), and semi-winter type as the primary rapeseed in China (Wei et al. 2017).

In Canola, the proportions of the unsaturated fatty acids, oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) are impacted by fatty acid desaturase 2 (FAD2) and FAD3.
Recently, scientists used genomic editing technology based on CRISPR/Cas9 system to mutate all the copies of fatty acid genes (BnaFAD2), creating novel allelic variations in oleic acid and other fatty acid levels (Huang et al. 2020). Thus, new canola varieties with high oleic acid content, which has tremendous health benefits including a reduction of cardiovascular disease (Jones et al. 2014). On the other hand, canola protein isolate has been suggested as an alternative to other proteins for human food due to a balanced amino acid profile (Tan et al. 2011). Nonetheless, canola seeds contain a high proportion of phytic acid, which is considered as anti-nutritive for monogastric animals including humans due to its adverse effects on essential mineral absorption (Anwar et al. 2015; Wickramasuriya et al. 2015). CRISPR/Cas9 technology was successfully used to knock out genes controlling the accumulation of phytic acid in canola seeds (Sashidhar et al. 2020), thereby improving the nutritional value of canola seeds.

9. CRISPR/Cas9 for Improved Soybean Oil Quality and Early Flowering

Soybean (Glycine max) is an important crop with abundant sources of protein and oil for human food and animal feed. Traditional soybean breeding strategies are insufficient to cope with the increasing demands for soybean products in challenging environment fluctuations. Therefore, it is essential to introduce fast, accurate and efficient breeding strategies to cultivate better varieties, especially those with traits involved in yield, quality and stress tolerance or resistance (Gao 2018).

Since the completion of soybean genome sequencing project, a major challenge and research priority facing soybean researchers is to characterize the function of 46-56 thousand annotated genes (Bai et al. 2020). Transgenic technology is a valuable method for functional genomic research and genetic improvement of crop, but its application in soybean has been hindered by the recalcitrant nature of soybean to transformation via Agrobacterium. Fortunately, new genome editing technology has brought opportunities to address above challenges. Using CRISPR/Cas9 technology, scientists recently mutated two genes (GmFAD2–1A and GmFAD2–1B) to produce a high oleic, low linoleic and α-linolenic acid phenotype in soybean (Do et al. 2019). Similarly, in previous study, scientists (Demorest et al. 2016) reduced the polyunsaturated fats
by editing the genes \textit{FAD2-1A} and \textit{FAD2-1B}, using another gene editing technique, TALEN (transcription activator-like effector nuclease).

Soybean is a long-season crop, and as a result it has narrow geographical adaptative region. Using CRISPR/Cas9 technology, scientists generated precisely targeted mutations in \textit{E1} gene controlling soybean flowering (Han et al. 2019). The truncated E1 protein in resulting mutants disinhibited \textit{GmFT2a/5a} leading to their increased expressions, resulting in early flowering. Practical applications of new gene editing technologies to soybean breeding are slowly emerging. A gene edited soybean oil with an altered fatty acid profile has just hit the market in USA (https://www.the-scientist.com/news-opinion/gene-edited-soybean-oil-makes-restaurant-debut-65590).

10. Improving Fiber Quality and Seed Oil Composition in Flax using Gene Editing

Flax (\textit{Linum usitatissimum}) is grown for its cellulose-rich fibers and seeds, which can be ground into a meal or turned into linseed oil. The long bast fibers are traditionally used in the textile, automobile, and construction industries (Baley et al. 2006; Kymäläinen and Sjöberg 2008). The use of plant fibers in various innovative fields has become very popular as they are environmentally friendly and biodegradable. Each fiber-consuming industry has special demands on the properties of fiber, which depend on morphological and anatomical characteristics, as well as on the structure and proportions of fiber bundle chemical constituents (Akin 2013). To manipulate these quality parameters, it is necessary to understand their genetic basis, and identify the genes controlling the target traits. Recently, RNA sequencing analysis identified 2666 genes with bast fiber-specific expression in flax (Mokshina et al. 2020). Proteins of the TIR-NBS-LRR class that are encoded by genes with fiber-specific expression, \textit{LusRAD-6} transcription factor, as well as the genes involved in cellulose biosynthesis (\textit{LusFLAs}, \textit{LusLTPs}, \textit{LusEXPA8}, and \textit{LusKIN14H}), and RG-I metabolism (\textit{LusRGL6} and \textit{LusGT106}) were suggested as the most obvious candidates for general fiber development (irrespective of the stage) and for fiber cell wall thickening. These genes may serve as a target for molecular-genetic modulation of their expression. Using CRISPR/Cas9 technique, scientists could exclusively modulate bast fiber development, without influencing other tissues.
On the other hand, the wide use of flax seed oil is hindered by its high content of polyunsaturated fatty acids, particularly linolenic acid (18:3), which accounts for 30%–40% of seed oil (Shim et al. 2014). This compound makes flax oil more susceptible to oxidation and food products derived from this oil are more prone to rancidity (Fröhlich and Rice 2005). To address this deficiency in flax oil quality, efforts have been focused on increasing the content of the more oxidatively stable oleic acid by suppression of FAD2 genes for the D12 oleic acid desaturase that converts oleic acid to linoleic acid (18:2) and linolenic acid (18:3) (Hutcheon et al. 2010; Kang et al. 2011; Nguyen et al. 2013). This genetic modification increased oleic acid content while decreasing polyunsaturated fatty acid (18:2 and 18:3) content of seed oils (Nguyen et al. 2013). The recent advent of the highly efficient and facile CRISPR/Cas9 system for gene editing offers the opportunity to determine whether the oil composition of flax seeds could be favourably altered by knocking out the activities of a few or all of the six fatty acid desaturase 2 (FAD2) genes present in the genome of this allohexaploid plant (Hutcheon et al. 2010; Kang et al. 2011). If successful, this strategy would increase oleic acid content and lower the content of linoleic acid, linolenic acid and other long-chain polyunsaturated fatty acids.

11. Gene Editing could improve Maize Salt tolerance and Lodging Resistance.

Maize is an important crop worldwide, providing more than one-half of global calorie consumption (Schnable 2015). However, the global maize production is increasingly being challenged by diverse environmental stresses (Deinlein et al. 2014; Zuo et al. 2015), including soil salinity stress (Farooq et al. 2015). Soil salinity causes tremendous crop yield losses worldwide because high level of Na+ (salt) results in nutrient deficiency, retarded growth, and cell death. Soil salinization is commonly associated to land clearing by removal of deep root vegetation, thus accumulating more water and consequently raising the level of salty groundwater (Almeida et al. 2017).

Maize salt tolerance is a complex trait of distinct mechanisms (Munns and Tester 2008; Zhu 2016), occurring at cellular, subcellular and organ levels. In particular, under salt-affected soils the plant releases the excess of salt through its leaves to balance the potassium/sodium (K+/Na+) ratio, which maintains the plant healthy. This adaptative mechanism is controlled by
the up and downregulation of genes. Genetic and molecular analysis identified the gene ZmNC1 (ZmHKT1) as a major player in the regulation of maize leaf Na\(^+\) extrusion and salt tolerance (Zhang et al. 2018a). Therefore, the application of new gene editing techniques (e.g., CRISPR/Cas9) to ZmNC1 would help creating maize genotypes with improved salt tolerance. Similarly, the gene Zm00001d039279 was identified recently (Sandhu et al. 2020) and was proposed to be a strong target for gene editing for enhanced salt tolerance. Rodríguez-Kessler (2006) found that two genes, Zmodc and Zmspds2A, are involved in salinity tolerance in maize roots through the accumulation of polyamine and spermidine. The expression of these genes could also be modulated through gene-editing techniques to prevent the harmful effects of soil salinization and avoid yield losses.

Semi dwarf plants can greatly contribute to crop improvement, as reported for semi dwarf “green revolution” rice (Sasaki et al. 2002) and wheat (Peng et al. 1999). The CRISPR/Cas9-mediated genome editing tool was utilized to edit the endogenous maize gene ZmGA20ox3 (GRMZM2G368411), which resulted in semi dwarf phenotypes improving lodging resistance (Zhang et al. 2020). Hu et al. (2019) used the same technique to edit the Semi-Dwarf1 (SD1) gene in two elite landraces, which contain many desired agronomic traits such as tolerance to low phosphorous and broad-spectrum resistance to several diseases and insects. Mutations of SD1 confer shorter plant height for better resistance to lodging. Field trials demonstrated that the yield of the new lines was better than that of the wild type under modern cultivation and that the lines maintained the same desirable agronomic characteristics as their wild-type progenitors.

12. Tomato with Improved Bioactive Components

Lycopene is considered as a bioactive component for treating chronic diseases and lowering the risk of cancer and cardiovascular diseases (Li and Xu 2014; Pouchieu et al. 2014). Modulation of the expression of key genes in the lycopene metabolism pathway is an effective way to increase lycopene content. Most of studies on enhancing lycopene accumulation in tomato fruit by regulating the carotenoid metabolic pathway were mainly focused on the modification of individual genes, including a null mutation of the gene lycopene \(\beta\)-cyclase 2 (LCY-B2) (Ronen et
al. 2000), overexpression of the phytoene synthase 1 gene (PSY1) (Fraser et al. 2007), suppression of the 9-cis-epoxycarotenoid dioxygenase 1 (NCED1) gene (Sun et al. 2012) and the silencing of stay-green 1 (SGR1) (Luo et al. 2013). This process is laborious and time consuming.

Recently, multiplex CRISPR/Cas9 editing was successfully applied to regulate multiple genes associated with the carotenoid metabolic pathway of tomato, to increase the lycopene content (Li et al. 2018c). Sites were designed to target SGR1 for promoting the synthesis of lycopene, whereas LCY-E, LCY-B1, and LCY-B2 for catalyzing cyclisation of lycopene, and BLC. LCY-E to prevent the cyclisation from lycopene to α-carotene, and LCY-B1 and LCY-B2 to prevent the cyclisation from lycopene to β-carotene (Moreno et al. 2013; Ralley et al. 2016). This study provides the basis for acquiring new tomato varieties with improved agricultural traits. Japanese government has just approved a gene-edited ‘super tomato’ which contains four to five times more gamma-aminobutyric acid (GABA) than a regular tomato, an amino acid believed to aid relaxation and help lower blood pressure (https://www.isaaa.org/kc/cropbiotechupdate/).

After the fruit reaches the optimum edible stage, it will slowly deteriorate and lose its quality. Therefore, the regulation of fruit ripening has become the focus of many studies (Martín-Pizarro and Posé 2018). Many ripening genes have been edited with CRISPR/Cas9, including RIN (Ito et al. 2017; Ito et al. 2015), IncRNA1459 (Li et al. 2018a), SlORRM4 (Yang et al. 2017), and SlDML2 (Zhou et al. 2019). All these results pave the way for creating new varieties in which fruits will remain firm for relatively long time while preserving their organoleptic properties.

13. Preserving Organoleptic Properties in Potato and Eggplant

Polyphenol Oxidases (PPOs) catalyze the conversion of phenolic substrates to quinones, leading to the formation of dark-colored precipitates in fruits and vegetables (Mayer 2006). This process, known as enzymatic browning, is the cause of undesirable changes in organoleptic properties and the loss of nutritional quality in plant-derived products (Jukanti 2017). In potato (Solanum tuberosum L.), enzymatic browning is a serious problem for both, producers and the industry, because the tubers can be affected during harvest and post-harvest procedures such as shipping, storage, distribution and blanching (Bachem et al. 1994).
In potato, PPOs are encoded by a multi-gene family with different expression patterns. A genome-wide survey revealed nine \( \text{StPPO} \)-like genes (named \( \text{StPPO1} \) to \( \text{StPPO9} \)), with differential prevalence of ESTs found from different potato tissues (Chi et al. 2014). Recently, the CRISPR/Cas9 system was applied to induce mutations in the \( \text{StPPO2} \) gene, responsible for most of the PPO activity and enzyme content in tubers (Chi et al. 2014). Interestingly, this gene editing technique also led to the elimination of larger, specific fragments from the coding sequence as was previously reported in potato (Tuncel et al. 2019; Veillet et al. 2019).

Eggplant (\( \text{Solanum melongena} \) L.) berries are characterized by a remarkable content of phenolic compounds, represented mainly by chlorogenic acid (5-O-caffeoylquinic acid). Chlorogenic acid plays important therapeutic roles due to its antioxidant, antibacterial, hepatoprotective, cardioprotective, anti-inflammatory and anti-microbial properties (Naveed et al. 2018). However, in commercial varieties, the selection for berries with a reduced degree of browning in the flesh has resulted in the indirect selection of accessions with lower concentrations of phenolics (Jaime et al. 2007). Recently, scientists used CRISPR/Cas9 gene editing to simultaneously induce mutations in three \( \text{PPO} \) genes, \( \text{SmelPPO4} \), \( \text{SmelPPO5} \) and \( \text{SmelPPO6} \), which resulted in reduced fruit flesh browning while preserving the content of phenolics (Maioli et al. 2020). This work opens the way to the development of eggplant genotypes with low flesh browning while maintaining a high polyphenol content in the berries.

14. Vicine-free Faba bean Cultivars

Faba bean (\( \text{Vicia faba} \) L.) is an excellent source of plant-based protein (Crépon et al. 2010) and provides a balanced diet of lysine-rich protein, carbohydrates, fibre and phytochemicals (Köpke and Nemecek 2010). However, its use as a food crop has been restricted, primarily due to the presence of glycosides vicine and convicine (\( \text{v-c} \)) in the seed. Ingestion of \( \text{v-c} \) can cause favism in some individuals with a genetically inherited deficiency (Luzzatto and Arese 2018). Levels of \( \text{v-c} \) can be reduced by soaking the seeds in a weak acid solution prior to cooking, by boiling, roasting or microwave irradiation, or via alkaline extraction with acid precipitation. Genetic approaches to reducing \( \text{v-c} \) levels are challenging given that faba bean is an outcrossing species. Indeed germplasm has been identified that has a 95% reduction in \( \text{v-c} \) levels, but the biosynthetic
pathway responsible for v-c levels is still lacking (Khazaei et al. 2019). Breeding efforts to reduce v-c contents are ongoing, but a near zero level of v-c has not yet been achieved with conventional breeding and processing methods (Crépon et al. 2010; Pulkkinen et al. 2015).

The genetic analysis of v-c production in faba bean supports a single major gene (Khazaei et al. 2015). Recent studies have elucidated the biosynthetic pathway for the pyrimidine glycosides vicine convicine (Björnsdotter et al. 2020). The bifunctional riboflavin biosynthesis protein RIBA1, which is now termed VC1, catalyses a key step in v-c biosynthesis. Editing this gene with a targeted single DNA base change should be sufficient to generate faba bean cultivars that are nearly free from these anti-nutrients, providing a safe and sustainable source of dietary protein. Since there are several faba bean cultivars already available that express suitable agronomics, gene editing would provide a rapid approach to convert these cultivars to low v-c types.

15. Eliminating the off-flavour of pea protein

Canada leads the world in dry pea production with a global share of 32%. As the market for alternative sources of proteins is expanding rapidly, pea has received much attention as a potential leading source of plant-based proteins. Major industry players, such as Roquette, Cargill and Burcon are investing nearly half a billion dollars in the construction of pea processing facilities. Thus, there is great potential for further economic growth in Canadian pea. Pea protein isolates and concentrates are key ingredients in diverse plant-based foods. Despite the positive consumer sentiment, the adoption of pea as a mainstream source of proteins is affected by the characteristic “off-flavors” often described as green, beany, hay-like, metallic, and astringent. The off-flavors are caused by a mixture of volatile organic compounds, such as methoxypyrazines, alcohols, ketones, aldehydes, etc. and non-volatile saponins. The production of volatile compounds in pea is initiated by the conversion of polyunsaturated fatty acids into hydroperoxide forms of fatty acids, mainly catalyzed by lipoxygenases (LOX). A null mutant of LOX2 has been shown to produce decreased levels of volatile organic compounds with no compromises in seed yield and weight (Forster et al., 1999). The LOX genes are obvious targets for CRISPR/Cas9 gene editing. The non-volatile saponins are a group of amphipathic compounds
comprised of lipophilic triterpene and hydrophilic sugar moieties. The biosynthetic genes for saponins have been reported in the literature (e.g., β-amyrin synthase, C22 hydroxylase, UDP-glycosyltransferase; Morita et al., 2000; Sundaramoorthy et al., 2019). CRISPR/Cas9 gene editing can be used to remove or alter the levels of saponins by creating null mutants of its biosynthesis genes.

Summary

Domestication and plant breeding has led to high yielding crop varieties which are adapted to local growing conditions. However, the growing human population faces a number of agricultural challenges including climate change, changes in abiotic stress and biotic stress along with loss of arable land and a demand for more sustainable and precise agricultural practices. Many crop traits have been fixed through initial waves of domestication, and in this review, we discussed several possibilities to generate another wave of important traits. CRISPR/Cas based gene editing provides a means by which we can create naturally occurring allelic variants without the constraints of traditional introgression breeding. In addition, we can now create new desirable genetic variants and counteract some of the loss of allelic diversity due to selective breeding. However, gene-editing should not be interpreted as a replacement for plant breeding. It is a tool of plant breeding, in that the technology generates genetic variation to which selection is applied. Indeed, the variation induced by gene-editing may not be the only strategy available to the plant breeder, nor will it negate the need for the plant breeder to evaluate the phenotypic outcome, or the stability of trait expression in a range of environments.

There still remains some challenges for routine application of gene editing in plant breeding. One of the biggest hurdles for the application of plant gene editing technologies is the need for a repeatable delivery method into totipotent cells, which could be widely applied to diverse plant species, especially recalcitrant species like pulse crops. Secondly, studies have shown that gene editing can lead to “off-target” mutations. However, as gene-editing technology has evolved, improvements are being seen in specificity, precision and off-target effects, editing capabilities, and ease of use in target organisms. Thirdly, functional genome annotation is critical to the success of gene editing experiments. Functional genomics research annotates the “role” of each
gene and it is necessary to bridge the gap between genotype and phenotype. Functional annotation is in most cases, a prerequisite to gene editing experiments. It allows selection of gene targets for editing that will have a high probably to impact trait response. It also provides clues as to the impact gene editing may have on gene function. Fortunately, the genome sequences of many important agricultural crops have become available, and functional annotation is becoming more routine. Gene editing provides an exciting opportunity to blend functional gene characterization with applied plant breeding. Lastly, the regulatory framework surrounding gene edited plant lines will impact how and where this technology is realized. Regulations that promote the use of gene editing are emerging in several countries, which will pave the way for programs to design strategies for optimal use of the technology to support cultivar development.
References


Do PT, Nguyen CX, Bui HT, Tran LTN, Stacey G, Gillman JD, Zhang ZJ, Stacey MG (2019) Demonstration of highly efficient dual gRNA CRISPR/Cas9 editing of the homeologous GmFAD2–1A and GmFAD2–1B genes to yield a high oleic, low linoleic and α-linolenic acid phenotype in soybean. BMC Plant Biology 19:311


Ralley L, Schuch W, Fraser PD, Bramley PM (2016) Genetic modification of tomato with the tobacco lycopene β-cyclase gene produces high β-carotene and lycopene fruit. Zeitschrift für Naturforschung C 71:295-301


Sashidhar N, Harloff HJ, Potgieter L, Jung C (2020) Gene editing of three BnITPK genes in tetraploid oilseed rape leads to significant reduction of phytic acid in seeds. Plant Biotechnology Journal 18:2241-2250


and hexaploid wheat through wider and longer grains. Theoretical and Applied Genetics 129, 1099-112.


Ujjal Kumar N, it, sup, gt, It, sup, gt, Hoy-Taek K, It, sup, gt, It, sup, gt, Khadiza K, It, sup, gt, It, sup, gt, Jong-In P, It, sup, gt, It, sup, gt, Kwon-Kyoo K, It, sup, gt, It, sup, and Ill-Sup N, It, sup, gt, It, sup, gt (2016) Modification of Fatty Acid Profiles of Rapeseed (&lt;italic&gt;Brassica napus&lt;/italic&gt; L.) Oil for Using as Food, Industrial Feed-Stock and Biodiesel. Plant Breeding Biotech 4:123-134


Zhou L, Tian S, Qin G (2019) RNA methylomes reveal the m<sup>6</sup>A-mediated regulation of DNA demethylase gene SIDML2 in tomato fruit ripening. Genome biology, p 156


